Serological detection of antibodies against *Salmonella* polysaccharides in ELISA employing a new method for coupling of polysaccharides

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Abstract

The ELISA (enzyme-linked immunosorbent assay) known as the mix-ELISA is used extensively for the screening of pig serum and meat juice samples for the presence of antibodies against *Salmonella* Typhimurium and *Salmonella* Infantis which are the most important *Salmonella* types occurring in Danish pig herds. Lipopolysaccharide (LPS) from *S.* Typhimurium and *S.* Choleraesuis is used as coating antigens in this assay. We undertook an effort to develop a robust and standardized version of this assay based on covalent coupling of antigenic *Salmonella* lipopolysaccharide-derived polysaccharides to microtiter plates by UV-irradiation. The polysaccharides were derived from lipopolysaccharide (LPS) by acid hydrolysis and then conjugated to a photochemically active anthraquinone (AQ) derivative which is capable of forming active radicals when exposed to UV-irradiation. The polysaccharide-AQ (PS-AQ) conjugate could then be activated by UV-irradiation and coupled covalently to the polymer plastic surface of the microtiter plates. Polysaccharides derived from *S.* Typhimurium and *S.* Choleraesuis lipopolysaccharides, representing the O-antigens 1, 4, 5, 6, 7, and 12, were used. Covalent coupling of polysaccharides by this technique ensures a regiospecific binding that does not influence the antigenicity of the polysaccharide antigen, as the antigen is bound to the plastic surface through the AQ part of the AQ-polysaccharide conjugate. In addition, conserved LPS domains such as lipid A which may give rise to cross-reactivity is avoided. Plates coated by this technique allow the employment of harsh assay conditions, such as extensive washing procedures and buffers with high salt concentrations when used in ELISA. The PS-AQ ELISA repeatedly had OD-values closely corresponding to those of the conventional mix-ELISA. The obtained functional polysaccharide surface was shown to be reproducible, with very low inter- and intra-plate variation in ELISA and was stable at room temperature for more than 6 months.

Introduction

Bacterial lipopolysaccharides (LPS) are very useful as antigens in serological assays due to their highly specific antigenicity (1). However, LPS can be difficult to coat, especially when using mixtures of two or more types of LPS by conventional passive coating procedures due to its amphiphilic structure, which may result in a non-uniform and non-reproducible antigen surface (2). This can be overcome by using a newly developed method using polysaccharide (PS) instead of LPS (3).

By this method the toxic lipid A part of LPS is cleaved from the antigenic PS part by mild acid hydrolysis and PS is coupled regiospecifically (through the KDO-residue of PS) to a photoreactive AQ-derivative and subsequently coupled to the microtiter plate by UV-irradiation (4). By this procedure the PS-AQ antigen is bound covalently to the microtiter plate.

We present here a summary of a serological application of this method in which a mixture of *S.* Typhimurium PS-AQ and *S.* Choleraesuis PS-AQ was photochemically coupled to microtiter plates (PS-AQ ELISA). The plates are designed as the conventional mix-ELISA (5) for serological monitoring of pigs; the combination of the two antigens will detect appoximately 95% of the *Salmonella* serotypes occuring in the Danish pig production (5). The methodology can be applied to PS from other *Salmonella* serotypes as well as to PS from other Gram-negative bacteria.

Materials and methods

S. Typhimurium and *S.* Choleraesuis were cultivated by fermentation and LPS was extracted by aqueous phenol. The LPS was subsequently partially hydrolyzed in acetic acid and extracted with chloroform/methanol yielding the PS. The purified PS was conjugated to AQ forming a PS-AQ conjugate. The antigenicity of the LPS, PS and PS-AQ preparations were tested in inhibition ELISA. The antigen preparations were incubated with the detecting pig serum against *S.* Typhimurium and *S.* Choleraesuis prior to incubation on the plate.

For the PS-AQ ELISA the two PS-AQ conjugates were covalently photocoupled to a microtiter plate (PolySorp, Nunc) by UV-irradiation (300-400 nm), see Fig.1. For the conventional LPS mix ELISA, microtiter plates were coated with LPS from the two *Salmonella* types.

ELISA was performed on the functional plates by addition of pig serum samples/pig reference sera, peroxidase-conjugated secondary antibodies against pig IgG and an OPD-hydrogenperoxide solution as chromogen-substrate (5).

Results

In general, inhibition ELISA showed that PS and PS-AQ preparations from both *S.* Typhimurium and *S.* Choleraesuis fully retained the antigenicity of the original LPS. When a mixture of these two types of PS-AQ was coupled to microtiter plates (Fig. 1) a functional PS-AQ surface was thus obtained. The performance of this surface was tested by ELISA (PS-AQ-ELISA) using pig sera and compared with identical testings on conventional, LPS (*S.* Typhimurium and *S.* Choleraesuis) coated microtiter plates (the mix ELISA (5)). Intra- and interplate variations were tested by analyzing serum samples in 16 duplicates/plate, calibrating against a reference pig serum (value equal to 100%). It was seen that the reproducibility of the PS-AQ coated microtiter plates was as good as or better than the reproducibility of the conventional LPS-coated microtiter plates and generally, in extensive tests of the overall assay variation between different batches of PS-AQ plates using a large panel of pig serum samples this was found to be at least equal to the conventional mix-ELISA. Also, the OD-values obtained were very similar between the two assays. The stability of the PS-AQ mix ELISA was tested over a period of 6 months at room temperature. It was seen that the *Salmonella* PS-AQ coated microtiter plates are stable at room temperature over the whole period showing performance within the overall assay variations.

Discussion

By this new technology we have shown that PS which cannot be passively coated onto plastic, can be easily immobilized covalently onto microtiter plates by UV-irradiation after conjugation to a photoreactive AQ-derivative. The PS retained full antigenicity after AQ-conjugation and binding to the plastic surface which thus represented a fully antigenic functionalized surface. With a large panel of pig sera the PS-AQ microtiter plates gave results that were indistinguishable from the results of the conventional mix-ELISA (5). They were furthermore very reproducible and showed a high stability (storage for more than 6 months at room temperature). This avoids batch variations of antigen coated microtiter plates during serological surveillance over a longer period, thereby minimizing day to day variation.

Also the PS-AQ antigen is less toxic than the LPS, and the coating time is strongly reduced (20 minutes as compared to overnight for passive coating of LPS). Furthermore, the covalent binding of the PS-AQ antigen to the microtiter plates allows harsh assay conditions (e.g. washing procedures, specific buffers) thereby increasing the signal-to-noise ratio.

In summary, the PS-AQ surface obtained by this new technique shows the following properties:

a) the covalent bond between antigen and plastic is regiospecific as the AQ group is only bound to KDO residues, which ensures a specific orientation of the PS, b) the antigenicity of the specific O-antigen epitopes is fully preserved, and c) it can be stored at room temperature for at least 6 months.

The ELISA performed on these plates showed a high degree of reproducibility, including low day-to-day variation, low interplate and intraplate variations of the microtiter plates (6). The PS-AQ ELISA gives results on individual sera that are similar to the results of the same sera in the mix-ELISA. In addition, the removal of lipid A may lead to a lower degree of cross-reactivity arising from antibodies with specificity against common epitopes of lipid A.

These properties of the PS-AQ functional surface are optimal for use in serological detection of antibodies in serum. The PS-AQ ELISA is therefore a valuable tool for serological surveillance of herds over longer periods; for serological evaluation of experimentally infected animals; and for diagnostic monitoring (6). The methodology can be applied to PS from any other *Salmonella* serotype, as well as to PS from other Gram-negative bacteria.

References

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